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Growth Factors Mobilize CXCR4 Low/Negative Primitive Hematopoietic Stem/Progenitor Cells from the Bone Marrow of Nonhuman Primates

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ABSTRACT

The chemokine receptor CXCR4 is expressed by $CD34^+$ hematopoietic stem/progenitor cells (HSC/HPC). Several investigators have suggested that expression of CXCR4 may be an important characteristic of HSC/HPC. We studied the dynamic expression of CXCR4 during growth factor–induced mobilization of HSC in a clinically relevant nonhuman primate model, *Papio anubis* (baboons). We evaluated whether CXCR4 expression in HSC/HPC varies during steady-state hematopoiesis as well as during growth factor–induced mobilization. Peripheral blood stem cells from 5 baboons were mobilized with growth factors. During mobilization, there was a consistent stepwise increase in the proportion of peripheral blood $CD34^+$ cells that were $CXCR4^-$. The highest number of $CD34^+CXCR4^-$ cells appeared in the peripheral blood at the same time as the maximum number of assayable colony-forming cells. The cloning efficiency of the $CD34^+CXCR4^-$ population was 3-fold greater than that of $CD34^+CXCR4^+$ cells, and the frequency of cobblestone area-forming cells was 6 times higher in the $CD34^+CXCR4^-$ population in comparison to $CD34^+CXCR4^+$ cells. Furthermore, the most quiescent $CD34^+$ cells isolated on the basis of low Hoechst 33342 (Ho) and rhodamine 123 (Rho) staining (Ho^{Low}/Rho^{Low}) were highly enriched in the $CXCR4^{Low/-}$ cell population. Ex vivo incubation of mobilized peripheral blood $CD34^+$ cells with growth factors for 40 hours resulted in increasing numbers of cells expressing CXCR4. Peripheral blood stem cell grafts containing $CD34^+$ cells that consisted of predominantly $CXCR4^-$ cells were able to rapidly engraft lethally irradiated baboons. Because the overwhelming number of $CD34^+$ cells within the mobilized peripheral blood grafts were $CXCR4^-$ and were capable of rescuing lethally irradiated baboons, it seems unlikely that the expression of CXCR4 in vitro is an absolute requirement for HSC homing and engraftment. In summary, our data suggest the dynamic nature of CXCR4 expression on $CD34^+$ cells during growth factor–induced HSC/HPC mobilization. In addition, our data indicate that the lack of CXCR4 expression is possibly a characteristic of relatively more primitive HSC/HPC characterized by a higher proliferative capacity.

KEY WORDS

CXCR4 • Chemokine • Stem cells • Mobilization • Hematopoiesis

INTRODUCTION

Hematopoietic stem/progenitor cells (HSC/HPC) present within the bone marrow (BM) are capable of maintaining continuous production of mature blood cells throughout the life span of an animal [1]. During ontogeny, hematopoiesis occurs at different sites, with developmental stage-specific shifts from the yolk sac to the aorta-gonad-mesonephros region, liver, and spleen and then to the BM and thymus [2]. Mechanisms orchestrating these shifts in hematopoietic activity are not well understood. The migratory ability of adult human HSC/HPC is highlighted during HSC transplantation, during which HSC/HPC seed and sustain donor-derived hematopoiesis in the BM of recipient after intravenous (IV) infusion [3]. In addition, recombinant human granulocyte colony-stimulating factor (rhG-CSF) administration results in the mobilization of HSC/HPC from BM to the peripheral blood (PB) [4,5]. A number of adhesive interactions between progenitor cells and BM stromal cells or extracellular matrix proteins have been

hypothesized to play a role in HSC/HPC trafficking [6]. The most compelling model for disruption of the normal processes governing HSC/HPC localization is provided by mice genetically engineered to be deficient in the expression of the chemokine receptor CXCR4 or its ligand, SDF-1 [7,8]. These animals are characterized by normal hematopoiesis within the liver but defective definitive hematopoiesis within the marrow of the developing rodents of this phenotype [7,8]. Several studies have suggested the variability of CXCR4 expression on mobilized PB (MPB), and this contradicts direct correlation of CXCR4 expression on HSC/HPC with homing and engraftment [9-11]. Similarly, cell-surface phenotype CD34, which is widely used to enumerate and enrich HSC/HPC, has been shown to vary with developmental stage [12]. Furthermore, Rosu-Myles et al. [13] and others have recently documented that both the $CXCR4^-$ and the $CXCR4^+$ population of human HSC from different tissues, including cord blood, were capable of hematopoietic engraftment when transplanted into nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice, indicating that surface expression of CXCR4 detected in vitro is not an absolute requirement for HSC homing [13,14]. Diminished CXCR4 expression by MPB $CD34^+$ cells was attributed to the internalization of the CXCR4 molecule, which can be quickly re-expressed upon exposure to growth factors [14]. Despite the variability of CXCR4 expression on human MPB $CD34^+$ cells, MPB grafts have been associated with more rapid hematopoietic engraftment [15]. In summary, whether the expression of CXCR4 is a characteristic of HSC/HPC remains unclear. Because important functional differences are known to exist between HSC/HPC of large animals and rodents [16-20], we attempted to study the dynamic changes of expression of CXCR4 on $CD34^+$ cells during growth factor–induced (recombinant human stem cell factor [SCF] and G-CSF or G-CSF alone) mobilization of HSC/HPC. During growth factor administration, we studied sequentially the distribution of $CXCR4^+$ cells in the BM and in the PB. In addition, we investigated whether there is a functional difference between $CD34^+CXCR4^-$ cells and $CD34^+CXCR4^+$ cells during steady-state hematopoiesis, as well as in MPB.

MATERIALS AND METHODS

Animals

Five healthy juvenile baboons (*Papio anubis*) weighing 9 to 10 kg were used for this study. The animals were housed under conditions approved by the Association for the Assessment and Accreditation of Laboratory Animal Care. The studies were performed under protocols approved by the Animal Care Committee of the University of Illinois at Chicago. Animals were provided with water, biscuits, and fruits throughout the study.

Leukapheresis

Leukapheresis was conducted after 5 to 8 days of growth factor (rhG-CSF alone or rhG-CSF and recombinant human SCF) treatment, depending on the total PB white cell counts, by using previously described protocols with little modification [16,21]. The animal's peripheral veins were cannulated with an 18-gauge Angiocath or a 16-gauge Teflon (DuPont, Wilmington, DE) catheter after the animal was anesthetized.

A Cobe Spectra (Cobe, Lakewood, CO) leukapheresis unit was used to collect the stem cell grafts. Leukapheresis was performed at a rate of 40 mL/min and a bowl speed of 900 to 950 rpm for 4 hours. The leukaphereses were performed with animals under ketamine sedation (10 mg/kg body weight intramuscularly) followed by thiopental (25 mg/kg body weight) induction and general oral endotracheal anesthesia (isoflurane 1%-2%). A Cobe Spectra leukapheresis unit was primed with approximately 180 mL of irradiated whole blood obtained from another ABO-compatible baboon blood donor. Venous access for both the draw and return lines was obtained by a surgical cut-down of the right femoral vein, where a double-lumen 7F pediatric hemodialysis catheter (Medcomp, Harleysville, PA) was inserted. Before leukapheresis, each animal was anticoagulated with heparin (50 U/kg) administered as an IV bolus after cannulation, followed by a continuous IV infusion at a rate of 10 U/kg/h). Acid citrate dextrose (Baxter/Fenwal, Deerfield, IL) was administered via continuous IV infusion at the rate of 1 mL/25 mL of whole blood entering the machine. Flow rates of approximately 20 to 25 mL/min were obtained to process approximately 3 blood volumes during the period of leukapheresis. The leukapheresis product was collected into a single plastic blood collection bag (Baxter) containing 20 mL of acid citrate dextrose.

BM Collections

BM samples were obtained from the iliac crests of animals after ketamine (10 mg/kg) and xylazine (1 mg/kg) sedation. The collection and separation of BM cells, PB cells, and cells within the leukapheresis product were performed as previously described [16]. The heparinized BM, PB, or LPs were diluted 1:8 in phosphate-buffered saline, and the mononuclear cell (MNC) fraction was obtained by centrifugation over 60% Percoll (Pharmacia, Uppsala, Sweden) at 500*g* for 30 minutes at room temperature.

Selection of $CD34^+$ Cells and Staining for CXCR4

A MNC fraction obtained by density gradient centrifugation was selected for $CD34^+$ cells by magnetic activated cell sorting (MACS; Miltenyi Biotec, Auburn, CA) by means of mouse immuno-

globulin (Ig)M monoclonal antibody (mAb) 12-8 (CD34; a gift of Dr. Robert G. Andrews, Fred Hutchinson Cancer Research Center, Seattle, WA) and anti-mouse IgM microbeads (Miltenyi Biotec), as described previously [16]. The positively selected cells were checked for purity by using donkey anti-rat IgG, an antibody against the isotype of microbeads used for CD34 selection with the MACS system. Anti-CXCR4 (clone 12G5; Pharmingen, San Diego, CA) or a matched isotype control was used to detect CXCR4 expression on both PB and BM by using either a FACSVantage or a FACSCalibur (Becton Dickinson, San Diego, CA). To confirm that the mAb against human CXCR4 cross-react with baboon blood cells, anti-CXCR4 mAb was titrated using 0 to 60 μL per test on baboon PB or BM cells and compared with that of human PB MNCs. When 20 μL of the anti-CXCR4 was used, the percentage of $CXCR4^+$ cells detectable among $CD34^+$ cells in baboon BM was comparable to that of $CD34^+$ cells that were stained to $CXCR4^+$ in the human PB (data not shown). In some experiments, another mAb, K6.1 (gift of the Naval Medical Research Institute, Bethesda, MD), a murine IgG2a, which also recognizes the analogous baboon CD34 epitope, was also used. Fluorescence-activated cell sorting was performed with FACSVantage. Subpopulations of $CD34^+$ cells were obtained on the basis of the presence or absence of CXCR4 expression. These cells were subsequently used for functional studies. The photomultiplier tube voltages were adjusted to compensate for the overlap of the fluorescein isothiocyanate and phycoerythrin emission spectra.

Hoechst/Rhodamine Staining

The fluorescent dyes Hoechst 33342 (Ho) and rhodamine 123 (Rho) (Molecular Probes, Eugene, OR) were used to obtain subpopulations of $CD34^+$ cells enriched for primitive hematopoietic progenitors, as described previously [16]. $CD34^+$ cells were suspended at the concentration of 10^6/mL in 0.1 μg/mL Rho and incubated in the dark for 30 minutes at 37°C. The cells were then centrifuged and resuspended in 10 μmol/L Ho and incubated at 37°C for 1 hour in the dark. The cells were washed twice in ice-cold phosphate-buffered saline containing 0.2% bovine serum albumin (Sigma Chemical Co., St. Louis, MO) and kept on ice until sorting. Cell sorting was performed with a FACSVantage. Green fluorescent pulses (Rho) were collected through a fluorescein isothiocyanate 530-nm filter with a bandwidth of 15 nm. UV emissions (Ho) were reflected by a 440 dichroic long-pass mirror and collected by a 424DF44 filter. Cells were sorted at a rate of 2000/s and collected in polypropylene tubes in media containing 20% fetal bovine serum (FBS; Hyclone, Logan, UT).

$CD34^+$ cells that had the least amount of DNA (Ho^{Low}) were further arbitrarily subdivided into Rho low (Rho^{Low}), Rho intermediate (Rho^{Int}), and Rho high (Rho^{Hi}) populations, as previously described [16]. Then $CD34^+$ cells were sorted for Ho^{Low}/Rho^{Low} cells. In addition, after Ho/Rho staining, cells were re-stained for CXCR4 and its matched isotype control.

Colony-Forming Cell Assays

Colony-forming cells (CFC) were assayed under standard conditions in semisolid media as previously described [16]. Briefly, 1×10^3 to 2×10^3 cells were plated in replicate cultures containing 1 mL of Iscove modified Dulbecco medium (IMDM) with 1.1% methylcellulose, 30% FBS, and 5×10^{-5} mol/L 2-mercaptoethanol (Methocult; Stem Cell Technologies, Vancouver, Canada), to which a cocktail of growth factors including 100 ng/mL recombinant human SCF, 100 ng/mL Flt3 ligand, 10 ng/mL interleukin (IL)–3, 10 ng/mL IL-6, 10 ng/mL granulocyte-macrophage colony-stimulating factor (all purchased from R&D Systems, Minneapolis, MN), and 5 U/mL of erythropoietin (a gift of Amgen, Inc.) were added. The cells were plated onto 35-mm tissue culture dishes (Costar, Corning Inc., Corning, NY), and after 14 days of incubation at 37°C in a 100% humidified atmosphere containing 5% CO_2, the colonies were scored with an inverted microscope by using standard criteria.

Cobblestone Area-Forming Cell Assays

The ability of primitive HSC/HPC to form cobblestone areas (CA) in long-term marrow cultures has been used as an in vitro surrogate HSC assay [21,22]. Baboon cobblestone area-forming cells (CAFC) give rise to undifferentiated, uniformly sized, round refractile cells arranged in a compact manner when cocultured with murine stromal fibroblasts in the presence of human cytokines for 5 weeks [16]. BM $CD34^+$, $CD34^+CXCR4^-$, and $CD34^+CXCR4^+$ cells were plated in limiting dilution in flat-bottomed 96-well plates (Costar, Corning Inc.) onto confluent, irradiated (7000 cGy) monolayers of the murine stromal fibroblast line M2-10B4 (a gift of C. Eaves, Vancouver, Canada). Each well contained 200 μL of a 50:50 mixture of IMDM and RPMI with 10% FBS. A cocktail of growth factors including 100 ng/mL SCF, 100 ng/mL leukemia inhibitory factor (a gift of Amgen), 50 ng/mL IL-3, 50 ng/mL IL-6, and 50 ng/mL granulocyte-macrophage colony-stimulating factor was added to these assays. The cytokine cocktail has been previously shown by our laboratory to be op-

timal for the development of baboon CA (data not shown). The cultures were fed weekly by replacement of half of the culture volume with fresh medium containing the above cytokines at 2 times the previously defined concentration. After 5 weeks of culture in a humidified incubator at 37°C containing 5% CO_2, the number of CA was scored with an inverted microscope by using standard criteria [23]. The CAFC frequency was computed by using minimization of χ by regression to the cell number at which 37% of wells were negative for CA formation, with 95% statistical precision [21–23].

Ex Vivo Culture of MPB CD34$^+$ Cells

Baboon CD34$^+$ cells enriched by MACS column were cultured in the presence of SCF (100 ng/mL) and G-CSF (20 ng/mL) supplemented with 10% FBS in IMDM. After 40 hours of incubation at 37°C in 5% CO_2, cells were harvested and stained for CD34 and CXCR4. The cells were acquired by FACSCalibur and analyzed with Cell Quest software (Becton Dickinson). At least 10 000 events were acquired for analysis.

Peripheral Blood Stem Cell Transplantation

Two weeks before transplantation, recipient animals were fitted with jackets and placed on a tether system, as previously described [20]. One week later, central venous catheters were placed in the jugular and femoral veins by surgical cut-down. Four days before transplantation, a myeloablative dose of total body irradiation (TBI) was delivered by a Varian Clinac 2100EX linear accelerator (Varian Medical System, Palo Alto, CA) by using a 6-MV photon beam through 2 lateral portals in 8 fractions twice daily from both sides of the body at a total dose of 1000 cGy (125 cGy × 2/day × 4) 24 hours before stem cell infusion. After completion of TBI (day 0), the animals were infused with cryopreserved peripheral blood stem cell (PBSC) or BM grafts.

Statistical Analysis

Statistical significance was determined by paired Student t tests with significance at $P < .05$.

RESULTS

Number of CXCR4$^+$ Cells in the PB after Growth Factor Administration

To investigate the role of CXCR4 in mobilization of HSC/HPC, the kinetics of the appearance of CXCR4$^+$ cells and CFCs were studied in MNC obtained from PB after growth factor administration to baboons. We studied the kinetics of CXCR4$^+$ cells and CFC number in the PB for 20 days after growth factor administration. SCF was administered on days 1 to 3, after which G-CSF was injected in combination with SCF on days 4 to 8. The percentage of CXCR4$^+$ cells in the PB decreased gradually during the course of growth factor administration (Figure 1). The percentage of CXCR4$^+$ and the cloning efficiency of CFC (mean number of colonies/number of cells plated × 100) in the PB of 2 animals is plotted in Figure 1. On day 7 and day 10 after growth factor administration, the number of CXCR4$^+$ cells in PB MNC of the 2 experimental animals decreased to their lowest levels (Figure 1A). The decline of CXCR4$^+$ cells in the PB was inversely related to the increases in the number of PB CFC (Figure 1B). The number of CXCR4$^+$ cells started to increase after 8 days of growth factor administration, whereas the number of CFC was reduced to baseline levels within 20 days from the start of growth factor administration. To further investigate the association between the numbers of CXCR4$^+$ cells and the numbers of CFC, we studied the kinetics of alteration of CXCR4 and CFC in the PB before and after growth factor administration in 3 additional animals. During steady state, 67.66% ± 6.53% of MNC in the PB were CXCR4$^+$, but after growth factor administration, the number of CXCR4$^+$ cells in the PB declined to 24.4% ± 3.5% ($P < .05$; Table 1). However, the cloning efficiency of CFC in the PB MNC before growth factor administration was 1.2% ± 1.3% ($P < .05$), which increased to 8.1% ± 1.3% after growth factor administra-

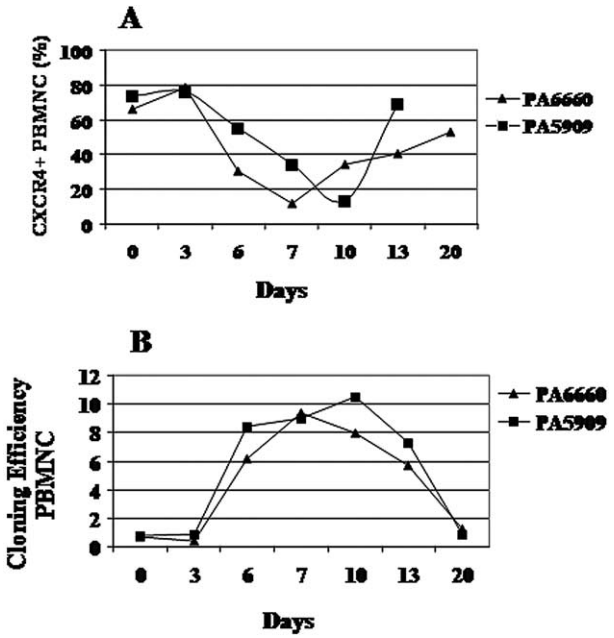

Figure 1. Expression of CXCR4 by PB MNC is inversely related to the number of PB CFC after growth factor–induced mobilization. During administration of recombinant human stem cell factor and rhG-CSF to juvenile baboons, PB MNC were analyzed at different time points to determine the kinetics of the appearance of CXCR4$^+$ cells by flow cytometry (A), as well as assayable progenitor cells in PB MNC (B). The kinetics of CXCR4$^+$ cells and the number of CFC are plotted from 2 different baboons (PA6660 and PA5909).

Table 1. *Increase in Peripheral Blood Hematopoietic Progenitor Cells Is Associated with a Decrease in CXCR4 Expression after Growth Factor Administration*

	CFC Cloning Efficiency		$CXCR4^+$ (%)	
Animal	PB	MPB	PB	MPB
PA5909*	1.4	9.4	ND	27
PA6660*	1.4	11.1	60	12
PA6661†	0.8	10.2	76	29
PA6243†	ND	6.3	67	32
PA5957†	ND	3.6	ND	22
Mean ± SE	1.2 ± 1.3‡	8.12 ± 1.3‡	67.66 ± 6.53§	24.4 ± 3.5§

Cloning efficiency indicates the number of colonies obtained out of the number of cells plated × 100.

*SCF and G-CSF were used to mobilize HSC/HPC in these animals.

†G-CSF alone was used to mobilize HSC/HPC; PB indicates peripheral blood; MPB indicates mobilized peripheral blood obtained by leukapheresis.

‡$P < .0005$; §$P < .025$, paired t test.

tion (Table 1). It was clear from these findings that the number of assayable CFC increased when the number of $CXCR4^+$ cells declined in the PB. To examine which cell populations may be responsible for the increase in CFC after growth factor administration, we analyzed $CD34^+$ cells from PB both before and after growth factor administration. On average, 32% ± 4.3% of the $CD34^+$ cells were $CXCR4^-$ in the PB of steady-state animals (day 0; Table 2). After growth factor administration, the percentage of $CD34^+$ that were $CXCR4^-$ increased to 76% ± 3.51% ($P < .05$; n = 5; Table 2). Steady-state BM had 41.0% ± 3.67% $CD34^+CXCR4^-$ cells, which increased to 51% ± 8.61% cells after growth factor administration. This modest increase in $CD34^+CXCR4^-$ cells in the marrow was not statistically significant ($P < .375$; n = 5; Table 2).

Absolute Number of $CD34^+CXCR4^+$ and $CD34^+CXCR4^-$ Cells in the MPB

Next we examined the absolute number of $CD34^+CXCR4^-$ cells in the PB in response to growth factor administration. Before growth factor administration, there were 2-fold more $CD34^+CXCR4^+$ cells than $CD34^+CXCR4^-$ cells in the PB (Table 3). Both $CD34^+CXCR4^-$ and $CD34^+CXCR4^+$ cells gradually increased from day 0 to day 6 in the PB after growth factor administration; however, after 6 days, the number of $CD34^+CXCR4^+$ cells started to decline while the number of $CD34^+CXCR4^-$ cells remained high (Table 3). By day 7, the ratio was dramatically altered: there were 7.3-fold more $CXCR4^-$ cells than $CXCR4^+$ cells among $CD34^+$ cells in the PB. This was due to a 51-fold increase in $CD34^+CXCR4^-$ cells and a 3.6-fold increase in $CD34^+CXCR4^+$ cells as compared with day 0 (before growth factor administration). The increase in the absolute number of $CD34^+CXCR4^-$ cells in the PB probably reflected the migration of this cell population from BM, proliferation of the existing $CD34^+CXCR4^-$ cells within the PB, or downregulation of CXCR4 expression after growth factor administration. Therefore, we intended to study whether the increase in the absolute number of $CD34^+CXCR4^-$ cells in PB was associated with a decrease of $CD34^+$ cells in the corresponding sample of BM. We studied 2 additional animals that received SCF and G-CSF for the mobilization of HSC/HPC. We observed 6- and 20-fold increases in the number of PB $CD34^+$ cells, respectively, in these 2 baboons (Figure 2). When we examined the corresponding BM sample of the same animal, the absolute number of $CD34^+$ cells increased 3-fold after growth factor administration in comparison to steady state. This increase

Table 2. *The Percentage of $CD34^+CXCR4^-$ Cells Is Altered in PB but Not in BM after Growth Factor Administration*

Tissues	No. Baboons Studied	$CD34^+CXCR4^-$
PB	3	32.3 ± 4.63*
MPB	5	75.6 ± 3.51*
BM	5	41.0 ± 3.67†
PBM	5	51.0 ± 8.61†

PB indicates peripheral blood; MPB, mobilized peripheral blood; BM, bone marrow; PBM, growth factor—primed bone marrow collected on the day of leukapheresis (corresponding sample of MPB).

*Significance by paired Student t test: $P < .005$.

†Significance by paired Student t test: $P < .375$.

Table 3. *Absolute Number of PB $CD34^+$ on the Basis of CXCR4 Expression*

Day	$CD34^+$ $CXCR4^+$ /μL	$CD34^+$ $CXCR4^-$ /μL
0	25	13
3	32	9
6	295	689
7	91	668
20	14	13

PB indicates peripheral blood; these data were obtained from a single animal who received SCF and G-CSF for PBSC mobilization.

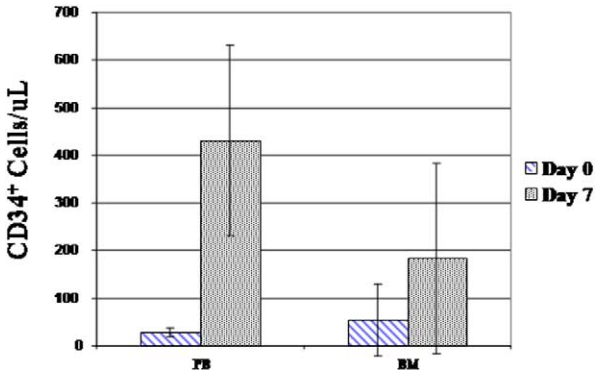

Figure 2. The absolute number of $CD34^+$ cells in PB and BM during steady state (day 0) and after growth factor administration (day 7). The data were obtained from 2 different baboons: PA6660 (A) and PA5909 (B). Each bar represents the number of $CD34^+$ cells per microliter of bone marrow or peripheral blood (mean ± SE) obtained from 2 separate animals.

in $CD34^+$ cells was associated with a corresponding increase in the absolute number of CFC both in the PB and BM (Figure 2).

$CD34^+Ho^{Low}/Rho^{Low}$ Cells Are Enriched for $CXCR4^{Low/-}$ Cells

To demonstrate the primitiveness of $CD34^+$-$CXCR4^-$ cells, we studied the expression of CXCR4 by $CD34^+Ho^{Low}/Rho^{Low}$ cells. This cell population presents a subpopulation of $CD34^+$ cells that are quiescent and enriched for CAFC during steady-state hematopoiesis [16]. A total of 61% of the BM $CD34^+$ cells expressed CXCR4, whereas only 13% of the Ho^{Low}/Rho^{Low} cells expressed $CXCR4^+$ (Figure 3). Therefore, 85% of $CD34^+Ho^{Low}/Rho^{Low}$ cells lacked surface expression of CXCR4, which was designated as $CXCR4^{Low/-}$. The mean fluorescence intensity of CXCR4 expression by $CD34^+Ho^{Low}/Rho^{Low}$ cells was 6.88, whereas presorted $CD34^+$ cells had a mean fluorescence intensity of CXCR4 of approximately 1001.

Clonogenic Potential of $CD34^+CXCR4^-$ Cells

To demonstrate the functional potential of $CD34^+CXCR4^-$ cells, $CD34^+$ cells from BM were isolated flow cytometrically on the basis of CXCR4 expression (Figure 4) and assayed for their ability to

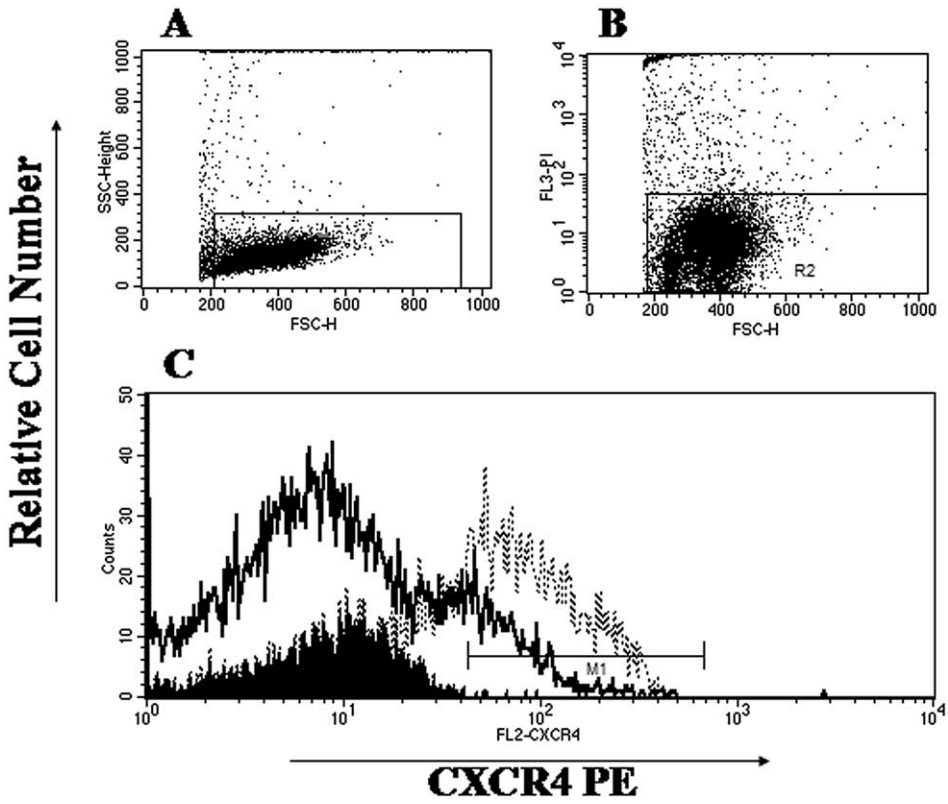

Figure 3. Subpopulations of $CD34^+$ bone marrow cells were sorted on the basis of Hoechst/rhodamine (Ho/Rho) staining as described previously [16]. Forward and side scatter profiles of $CD34^+Ho^{Low}/Rho^{Low}$ cells (A) and gating on propidium iodide–negative live cells (B) are shown. Gated live cells on the basis of the gate shown in (A) and (B) are presented as histograms (C). The dark filled histogram represents the isotype control, the dotted line histogram is an overlay representing expression of CXCR4 by $CD34^+$ cells before sorting, and the continuous line histogram is a overlay representing expression of CXCR4 by $CD34^+$ cells sorted flow cytometrically on the basis of Ho^{Low}/Rho^{Low}, the most quiescent and primitive population in baboons [16]. M1 was set on the basis of isotype control.

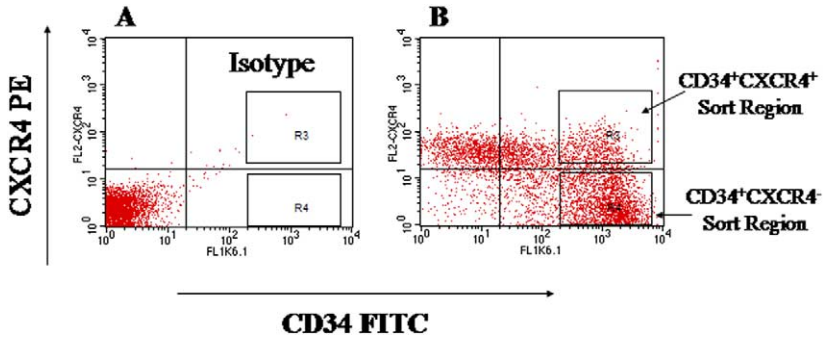

Figure 4. Bone marrow $CD34^+$ cells were sorted flow cytometrically on the basis of CXCR4 expression. The isotype for CXCR4 phycoerythrin is shown in (A), and the cursor lines in the dot plots are set according to the isotype control. The sort regions for $CD34^+CXCR4^+$ and $CD34^+CXCR4^-$ cells are shown in (B).

form hematopoietic colonies (CFC) in vitro. Then the CFC potential of $CD34^+$, $CD34^+CXCR4^+$, and $CD34^+CXCR4^-$ cells was individually determined. In Figure 5, we demonstrate that $CD34^+CXCR4^-$ cells contained more than 4-fold more CFC in comparison to the $CXCR^+$ population, including more mixed colonies in the $CXCR4^-$ population ($P < .025$), indicating their primitive nature. To further examine the content of these cells, we investigated whether $CD34^+CXCR4^-$ cells were also enriched for more primitive HPCs such as CAFC. CAFC assays were performed in limiting dilution to estimate the frequency of CAFC. We observed that the $CD34^+CXCR4^-$ cells contained 6-fold more CAFC than $CD34^+CXCR4^+$ cells (Figure 6). These findings clearly demonstrate that BM $CD34^+CXCR4^-$ cells are more primitive than $CD34^+CXCR4^+$ cells by virtue of their greater content of assayable CFC and CAFC.

Ex Vivo Culture of MPB $CD34^+$ Cells

The increase of the number of PB $CD34^+$-$CXCR4^-$ cells after growth factor administration could be attributed to 2 possibilities: growth factor treatment either results in reduction of CXCR4 expression by $CD34^+$ cells or is due to preferential mobilization of $CD34^+CXCR4^-$ cells. To further examine the role of growth factors on the expression of CXCR4, $CD34^+$ cells enriched from MPB or BM were cultured in vitro with G-CSF and SCF for 40 hours. On average, 12% of MPB cells were $CD34^+CXCR4^+$ before culture (0 hours), and after 40 hours of incubation, the numbers of $CD34^+$ cells expressing CXCR4 increased to 46% ($P < .05$; Table 4). One of the representative flow cytometric analyses of cells cultured ex vivo is shown in Figure 7. These data clearly showed that growth factor exposure in vitro resulted in increased expression of CXCR4 by $CD34^+$ cells.

MPB Cells Are Capable of Engrafting Myeloablated Baboons

When MPB cells mobilized by SCF and G-CSF were used as autografts for 2 myeloablated baboons, the $CD34^+$ cells comprising mostly $CXCR4^-$ cells resulted in a rapid pattern of hematopoietic engraft-

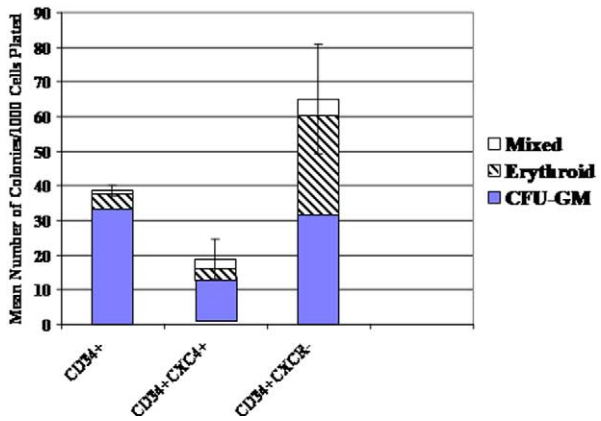

Figure 5. Content of hematopoietic progenitor cells within $CD34^+CXCR4^+$ and $CD34^+CXCR4^-$ cells obtained from steady-state baboon bone marrow. The bar graph represents the mean number of colonies ± SEM assayed in triplicate from 2 different baboons.

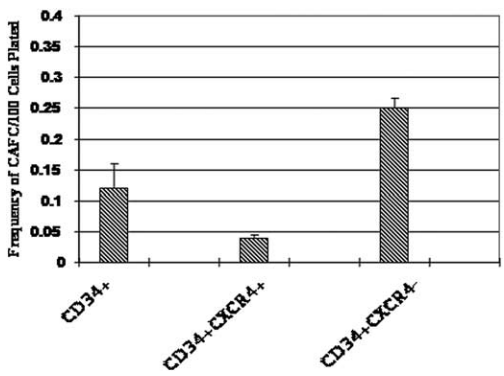

Figure 6. Distribution of CAFC frequency among different subpopulations of marrow $CD34^+$ cells based on CXCR4 expression from steady-state baboon bone marrow. The bar graph represents the mean ± SEM of the frequency of CAFC obtained from 2 different animals.

Table 4. *Ex Vivo Culture of CD34$^+$ Cells Results in an Increase of CXCR4 Expression*

Tissue	CD34$^+$CXCR4$^+$ (%) 0 h	40 h*
MPB†	12.0	44.0
MPB	22.0	44.0
MPB	3.0	37.0
MPB	11.0	59.0
Mean ± SE	12.0 ± 11.58‡	46.0 ± 4.64‡

*MPB CD34$^+$ cells were cultured ex vivo in the presence of SCF and G-CSF for 40 h.

†This animal was mobilized with SCF and G-CSF; all other cases shown here were mobilized with G-CSF alone.

‡Paired t test; $P < .005$.

Table 5. *Hematopoietic Engraftment after Transplantation of MPB and BM*

Animal	Day of Recovery after Transplantation: CD34$^+$ Cell Dose*	WBC >1 × 10^3/μL	Platelets >20 × 10^3/μL
PA6661†	3.5	10	15
PA6243†	2.4	12	16
PA6592‡	21.0	14	21
PA6663‡	6.6	14	21

Table 5 is part of previously published data. Reprinted with permission [23].

*Expressed as 10^6 cells per kilogram body weight.

†These animals received injections of G-CSF alone to collect the mobilized peripheral blood (MPB) cell graft.

‡These animals were not stimulated with growth factors, and bone marrow (BM) cells were used as a graft.

ment in comparison to other 2 animals who received autologous BM grafts (Table 5). Although the grafts were not selected, more than 85% of CD34$^+$ cells in PBSC grafts were CXCR4$^-$, whereas only 41% of CD34$^+$ cells in BM grafts were CXCR4$^-$ (Table 2). In addition, we did not observe any significant difference in the stability of engraftment over 60 to 100 days after transplantation in animals receiving either the MPB or BM grafts (data not shown). Despite a greater absolute number of CD34$^+$CXCR4$^+$ cells per kilogram body weight in BM grafts, MPB grafts engrafted faster. Indeed, the possibility of rapid re-expression of CXCR4 by CD34$^+$ cells in vivo after IV administration in baboons cannot be ruled out, because after ex vivo exposure to growth factors, MPB CD34$^+$ cells had an increase in the number of CD34$^+$ cells coexpressing CXCR4$^+$.

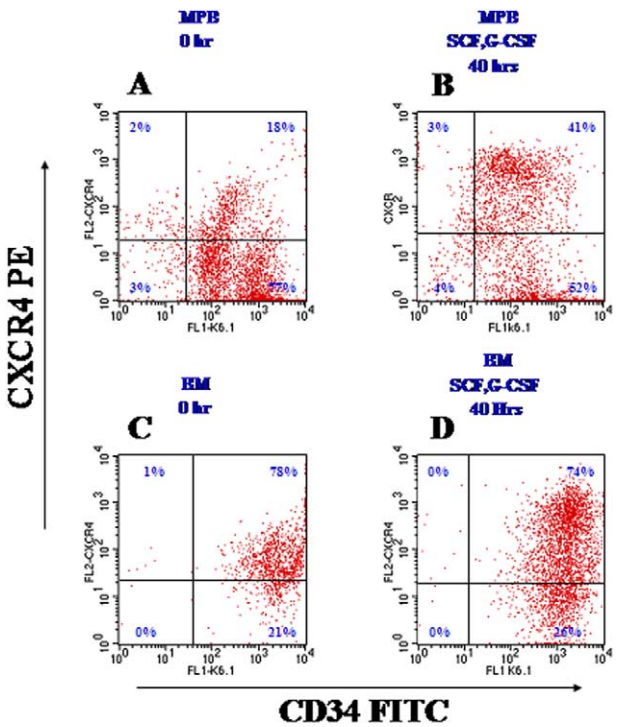

Figure 7. CD34$^+$ MPB and BM cells were cultured ex vivo in media supplemented with FBS containing G-CSF and SCF for 40 hours and examined flow cytometrically for expression of CXCR4 on CD34$^+$ cells. MPB cells are shown before (A) and after (B) culture. (C) BM before culture; (D) BM CD34$^+$ cells after culture. The cursor line on the dot plot was placed on the basis of matched isotype control. The percentage of each subpopulation of cells is shown in each quadrant.

DISCUSSION

MPB is increasingly being used as a source of HSC grafts in humans [15]. Rapid engraftment of hematopoietic cells after PBSC transplantation has been well documented [24,25]. The interaction between chemokines and their receptors has been thought to play a role in stem cell engraftment. It has already been shown that HSC/HPC, including CD34$^+$ cells, express CXCR4, which binds to its ligand SDF-1, expressed by BM stromal cells [12]. The analysis of CXCR4 in MPB CD34$^+$ cells has been reported by several laboratories with variable results [11,13,14]. In this study, we have shown that in steady state, BM CD34$^+$CXCR4$^-$ cells are the most quiescent and highly enriched for both primitive (CAFC) and more differentiated (CFC) hematopoietic progenitors. In addition, CD34$^+$ cells in PB after growth factor administration are enriched for HPCs in comparison to steady-state PB. Our findings clearly demonstrate that this increase in clonogenic cells in the PB is associated inversely with the number of cells expressing CXCR4. The kinetics of CXCR4 expression on PB MNC after an environmental stimulus (growth factors) in a clinically relevant large-animal model support the observation that CXCR4 expression by CD34$^+$ cells is a dynamic process. This finding is consistent with others with a murine model [14]. These CD34$^+$CXCR4$^-$ cells are more abundant in the steady-state BM as compared with steady-state PB. A net increase in the absolute number of

$CD34^+CXCR4^-$ cells in the PB during growth factor–induced mobilization was observed without a net reduction of the $CD34^+$ cell population in the BM. However, downregulation of CXCR4 by $CD34^+$ cells after in vivo growth factor administration is unlikely because ex vivo culture of MPB $CD34^+$ cells in the presence of growth factors resulted in an increase in the number of $CD34^+CXCR4^+$ cells. These findings are consistent with an earlier observation that exposure of $CD34^+$ cells to growth factors results in greater CXCR4 expression [10]. These findings led us to examine whether $CD34^+CXCR4^-$ cells are relatively more primitive as compared with a $CD34^+CXCR4^+$ population of cells. In vitro clonogenic assays provided evidence that marrow $CD34^+CXCR4^-$ cells are enriched for both primitive and more differentiated HSC/HPC in vitro. This is consistent with the finding of others showing that $CXCR4^-$ cord blood cells are capable of engrafting and producing multiple hematopoietic lineages when transplanted into the NOD/SCID mouse model [13]. Furthermore, in agreement with our hypothesis, Ishii et al. [26] have shown that human $CD34^+CXCR4^+$ cells from BM were completely devoid of myeloid, erythroid, megakaryocytic, and mixed CFCs yet possessed the potential to differentiate into lymphoid cells. These studies indicate that $CXCR4^+$ cells are actually committed lymphoid progenitors. By contrast, the same group showed that marrow $CD34^+CXCR4^-$ cells possess multilineage differentiative potential in vitro, suggesting that this cell population might resemble an HSC [26]. This group, however, did not perform an assay for more primitive progenitors (CAFC) but did demonstrate that $CD34^+CXCR4^+$ cells could be generated from $CD34^+CXCR4^-$ cells, indicating the likelihood that $CD34^+CXCR4^-$ cells are more primitive than $CD34^+CXCR4^+$ cells [26]. Therefore, the strategy suggested by others performing ex vivo culture to increase CXCR4 expression in vitro to enhance their homing potential warrants more careful evaluation because the expression of CXCR4 in our study is associated with differentiation of primitive HSC/HPC [10]. Furthermore, ex vivo cultured hematopoietic grafts have had limited engraftment potential because of discordance between phenotype and function with the lack of true expansion of engraftable cells [21,27].

We have previously demonstrated that baboon $CD34^+Ho^{Low}/Rho^{Low}$ cells represent a quiescent primitive $CD34^+$ cell subpopulation [16]. In our current study, adult baboon $CD34^+Ho^{Low}/Rho^{Low}$ marrow cells contained the highest number of $CD34^+CXCR4^{Low/-}$ cells during steady-state hematopoiesis. Our findings also suggest that growth factors possess the ability to mobilize the most primitive marrow $CD34^+CXCR4^-$ cells to the PB from the BM of baboons. In our studies, G-CSF was capable of resulting in an increase in the absolute number of $CD34^+CXCR4^-$ relatively primitive cells in the PB. It is interesting to note that Ishii et al. [26] observed that $CD34^+CXCR4^-$ cells expressed the receptor for G-CSF by reverse transcription-polymerase chain reaction but that $CD34^+CXCR4^+$ cells did not. These findings support the hypothesis that the $CD34^+CXCR4^-$ primitive population is possibly a more specific target population for G-CSF containing mobilizing regimens. Our findings demonstrate that $CD34^+$ cells that do not express CXCR4 are relatively more primitive and remain capable of mobilizing to the PB after growth factor administration. These data suggest that expression of CXCR4 by $CD34^+$ cells is a consequence of differentiation and that the role of this chemokine receptor in stem cell homing requires further investigation.

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